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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have demonstrated that (1) alcohol-naïve rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and preference which are highly correlated with acoustic startle amplitude determined before the initial access to alcohol, providing a prospective index of vulnerability to developing alcohol abuse, as well as insights into mechanism. We have also demonstrated that (2) suppression of noradrenergic signaling decreases alcohol drinking in rats with a history of traumatic stress, but not in rats without this stress history. This result informs clinical studies in which subjects are reported to exhibit variable responses to this treatment. In addition, we have demonstrated (3) that suppression of noradrenergic signaling at the time of traumatic stress decreases acquisition of increased voluntary alcohol drinking long after the stress, which provides a new model for preventive treatment (these results were presented at the 2016 Research Society on Alcoholism meeting). Accomplishment 1 has been published, 2 and 3 are in preparation for publication. All remaining proposed studies using rat models to address stress and PTSD mechanisms as targets for pharmacotherapy of PTSD and associated alcohol abuse are currently in progress as planned, with no changes in scope, although with some delays due to personnel changes and due to the need for some methodology refinements.

15. SUBJECT TERMS

PTSD, alcohol, ethanol, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse

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1. **INTRODUCTION:** Studies from our research group demonstrating that the well-characterized, safe, well-tolerated and FDA-approved α1-adrenergic receptor antagonist (AR), prazosin, is effective not only in treating combat post-traumatic stress disorder (PTSD) symptoms but in decreasing alcohol drinking in both human and rat studies provide much-needed breakthroughs in the development of effective pharmacotherapies for alcohol use disorders as well as for PTSD. However, much work remains to determine conditions in which this treatment to reduce noradrenergic hyperactivation will be effective, characteristics of individuals who are most likely to respond, and underlying mechanisms providing bases for additional treatments. Our immediate objective is to identify key variables in rat models that will inform and complement human studies, providing a powerful translational approach for most efficiently and rapidly developing and implementing effective new pharmacotherapies for alcohol use disorders and co-morbid PTSD.

2. **KEYWORDS:** alcohol, ethanol, PTSD, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse

3. **OVERALL PROJECT SUMMARY:** There are no significant changes in the project goals or studies planned.
   - **CURRENT OBJECTIVES** There are 3 objectives of this project, which have remained unchanged. In order to be consistent with the organization and section headings of the current SOW, these 3 major objectives continue to be presented here as SPECIFIC AIMS 1-3, although each of these specific aims now also are identified (in parentheses) as Objective 1, 2 or 3, for further clarity. Also consistent with the organization of the current SOW, Tasks 1-6 are discussed within these three SPECIFIC AIMS (Objectives), identified by bold text.
     - **SPECIFIC AIM 1 (Objective 1): Determine relationship of hyperexcitability, anxiety and α1-adrenergic receptor-mediated signaling to excessive voluntary alcohol drinking**, *providing information from rat models that will likely reveal especially promising bases for:*

     a) Prospectively identifying subsets of individuals who are highly vulnerable to developing alcohol use disorders (AUDs). **(TASK 1)**

     STATUS: We have demonstrated that alcohol-naïve rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and alcohol preference in an intermittent alcohol access (IAA) paradigm. These results are consistent with the central hypothesis for all other studies in this research project, i.e., that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to – or at least is associated with – development of increased alcohol drinking. This work was completed in year 1 and

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described in detail in the Year 1 progress report. The published paper is included in the appendix (Rasmussen and Kincaid, 2015).

b) *predicting who is most likely to respond to prazosin with decreased alcohol drinking.* **(This work was not assigned a separate specific TASK # in the SOW, but it is a component of several of the proposed experiments. To facilitate discussion, we include it here as TASK 1b).**

STATUS: Although our initial work suggests that high acoustic startle and increased anxiety-like behavior is associated with increased suppression of alcohol drinking in response to prazosin, as hypothesized, we are investigating responses in all alcohol access and PTSD-like conditions of this overall investigation, as planned. Consequently, resolution of this issue will not be finalized until all experiments are completed. As described in the original proposal, prazosin is being administered prior to voluntary alcohol drinking in rats that have been previously characterized for acoustic startle and anxiety-like behaviors in the differing experimental models used in these studies; we continue to evaluate whether prazosin treatment disproportionately decreases alcohol drinking in those rats with pre-existing or PTSD-induced high acoustic startle and high anxiety-like behavior. Nonetheless, an early result is already of great interest and likely clinical relevance. In an initial trial with the rat PTSD model used throughout these investigations (and described in detail in the proposal), 51 young male Wistar rats received either 10 seconds of inescapable footshock (traumatic shock; TS) or no shock (NS) followed by 4 weekly contextual reminders (R) of the TS or NS (but no further application of TS or NS). The rats were then provided 4 weeks of intermittent alcohol access (IAA, 24 h/day free choice between 20% alcohol *vs* water on 3 non-consecutive days/week) in order to establish stable elevated levels of voluntary episodic alcohol drinking. Alcohol access was then further restricted to 1 hour on each IAA day for the next 4 weeks. On 1 day of each week, each rat received an intra-peritoneal (IP) injection of either vehicle alone (VEH), the alpha-1 adrenergic receptor antagonist, prazosin (PRZ, 1.5 mg/kg), the beta-adrenergic receptor antagonist, propranolol (PROP, 5 mg/kg), or the combination of PRZ+PROP (1.5 mg/kg, 5 mg/kg) at 30 min before the 1 hour alcohol access period, with each rat receiving each of the 4 treatments in counterbalanced order over the 4 weeks. Results are shown in **Fig. 1**. In the rats that had not received TS ("Non-Stress" in the figure), there was not a significant change in alcohol intake in response to either drug alone or to the combination of drugs, even though the doses of each drug were the same as we have previously demonstrated to consistently decrease alcohol drinking in selectively-bred alcohol-preferring (P) rats derived from the Wistar strain. However, rats that had received a single application of TS at least 8 weeks before testing ("Traumatic Stress" in the figure) exhibited consistently decreased acute alcohol intake in response to prazosin treatment, and this suppression was enhanced by combining the

prasozin treatment with propranolol treatment (this enhanced suppression of alcohol drinking by addition of propranolol to prazosin treatment is consistent with our previous results in P rats). These results suggest that, in normal outbred rats voluntarily drinking alcohol in an IAA model, the response to reduction of noradrenergic activity by prazosin or prazosin+propranolol is dependent on history of previous stress. These results are clinically important because responses to prazosin treatment are not consistent among subjects, including among PTSD patients, in clinical investigations. The results thus suggest that history of prior high stress may
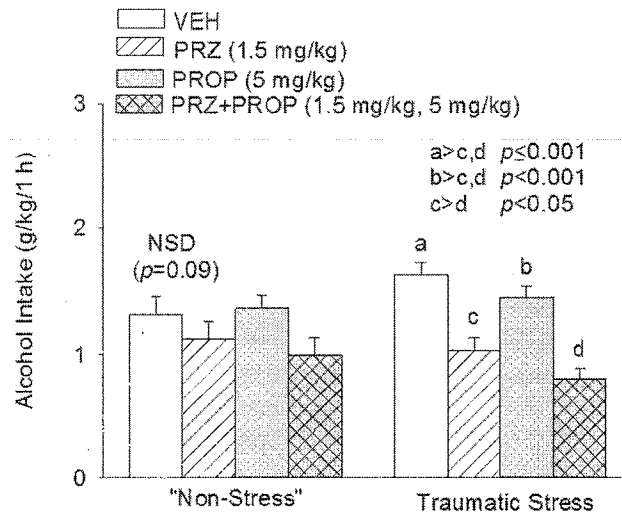

FIG. 1

determine response to pharmacotherapy that decreases noradrenergic signaling. In addition, this rat model may provide an especially effective preclinical model for further resolving the brain, neuroendocrine and behavioral mechanisms mediating this disparity in responses.

We will present this completed work at either the 2017 Annual Meeting of the Research Society on Alcoholism or the 2017 Fourth International Congress on Alcoholism and Stress. A corresponding manuscript is in preparation.

c) preventing initial acquisition of AUDs in prospectively-identified vulnerable individuals. (TASK 2)

STATUS: The central hypothesis of this experiment is that rats exhibiting high acoustic startle before the initiation of IAA will subsequently exhibit high IAA alcohol intake (as we have demonstrated in Task 1), and that continuous treatment to suppress noradrenergic signaling before and throughout IAA will prevent this acquisition of high alcohol drinking. This experiment thus requires continuous treatment of the rats during introduction of IAA. We had originally proposed to accomplish this with oral or intraperitoneal administrations 3 times each day. However, more recent reports suggested that prazosin could be administered with implantable osmotic minipumps to maintain prolonged (4 week) constant administration with minimal stress, so we changed the route of drug administration for this experiment (with prior approval at the time of the first annual report). Results are shown in **Fig. 2**. After each young male Wistar rat was characterized for acoustic startle response (ASR) and behavioral indices of anxiety, the rats were each implanted

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subcutaneously with an Alzet Osmotic Minipump delivering either vehicle alone (VEH; 50% DMSO, necessary to solubilize the PRZ in a high enough concentration to deliver 2 mg/kg/day for 28 days), prazosin (PRZ) 2 mg/kg/day), propranolol (PRO, 5 mg/kg/day), or prazosin+propranolol (P+P; 2 mg/kg/day, 5 mg/kg/day) and then provided IAA for 4 weeks (spanning 12 IAA sessions and the entire 4 week period that the Alzet pumps were designed to deliver constant drug administration). An additional non-treated (NTR) group of rats that did not receive implantation of an Alzet pump and did not receive infusion of either VEH or drugs likewise was provided IAA during the same 4 week period, in the same colony room. Rats receiving a pump delivering VEH consumed less (p<0.01) alcohol than the untreated NTR rats when the alcohol intake was determined either in the first hour of each IAA session (upper panel) or during the entire 24 hours of each session (lower panel), without significantly
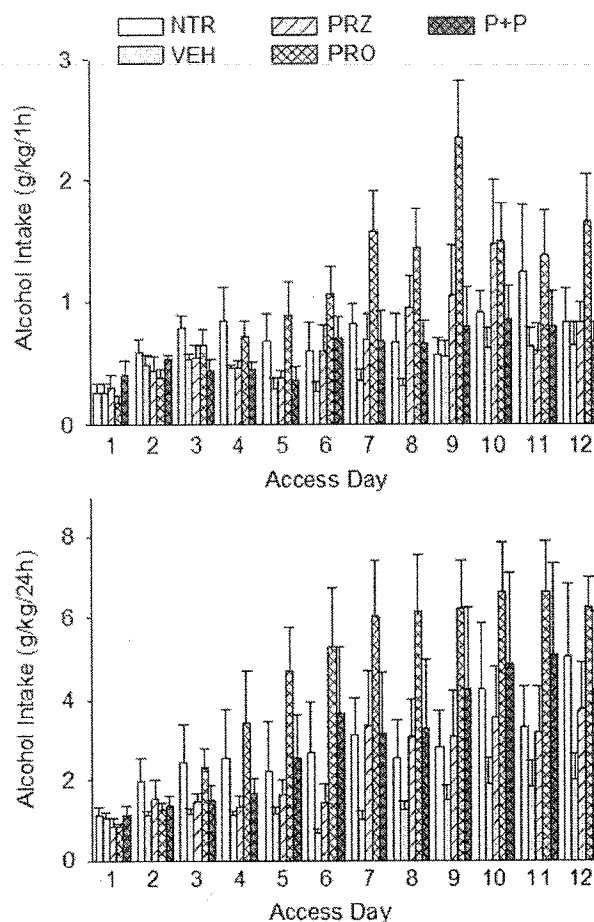

FIG. 2

consistent responses to PRZ or P+P treatment (compared to VEH). Consequently, there were two major problems. The first was that osmotic pumps delivering only VEH suppressed alcohol intake (compared to NTR rats), suggesting that either stress associated with the implanted pump or perhaps the administration of vehicle alone suppressed alcohol drinking. The second was that PRZ or P+P were ineffective in consisting altering the alcohol drinking. To address why the PRZ and P+P were ineffective, we examined the pumps upon removal after euthanasia at completion of the experiment, 4 weeks after implantation. 80% of the PRZ rats and 100% of the P+P rats, but none of the VEH or PRO rats, had pumps that were swollen, shown in **Fig. 3**. When we cut the pumps open, it was apparent that the PRZ had precipitated out of solution in the PRZ and P+P pumps and that the orifice through which the drug is delivered

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was completely clogged in each. The pumps from the PRZ and P+P rats contained a large amount of fluid with flocculent white precipitate whereas the pumps from VEH and PRO rats were
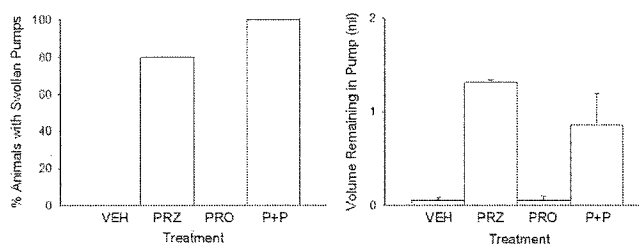
FIG. 3

essentially empty; i.e., the PRZ and P+P rats had clearly not received the intended drug treatment. Consequently, this study now will be repeated using the oral route of administration as originally proposed. In order to circumvent the known problem of prazosin short metabolic half-life that requires multiple daily treatments over the entire 4 week treatment period, we now propose to instead use the drug doxazosin, an analog of prazosin which is functionally essentially identical - with the same alpha-1 adrenergic receptor antagonist specificity - but which has a much longer half-life that allows once/daily administration in clinical applications. We have already reported that doxazosin suppresses alcohol drinking in rats comparably to prazosin, and doxazosin has recently been reported (by a group including my colleague and consultant, Dr. Murray Raskind) to suppress clinical symptoms of PTSD similarly to prazosin. In order to allow investigation of multiple doxazosin doses (as originally proposed for prazosin) we will investigate only VEH vs doxazosin treatments. So, although this study was completed, we propose to adjust the model to eliminate problems that we encountered and repeat the study.

Due to the unanticipated problems, the objective of this study has been 0% achieved, but the proposed minimal change in procedure should allow us to achieve it efficiently and 100%.

d) predicting who is most vulnerable to progression from voluntary to compulsive alcohol drinking. **(TASK 3)**

STATUS: This prolonged (28 week) study evaluates the hypothesis that prazosin will suppress high alcohol drinking even in rats that had exhibit high acoustic startle and associated anxiety-like behavior before IAA and which subsequently develop compulsive alcohol drinking (i.e., alcohol drinking that is maintained even after a distasteful adulterant – e.g., quinine - is added to the alcohol). This study is ongoing, as illustrated in **Fig. 4**. Male Wistar rats (n = 39) have received either continuous or IAA access to 20% alcohol *vs* water. As predicted, rats with continuous alcohol access have consumed less alcohol on a daily basis and also have exhibited lower alcohol preference, compared to rats receiving IAA (p<0.001). In week 15 of alcohol access, testing for response to quinine addition to the alcohol was initiated. However, on the first day of testing, the lowest concentration of quinine (0.01 g/l; previously demonstrated to not alter alcohol intake in rats exhibiting compulsive-like alcohol intake) suppressed alcohol drinking by all rats, providing no

evidence that compulsive alcohol drinking had yet been established. Consequently, we are extending
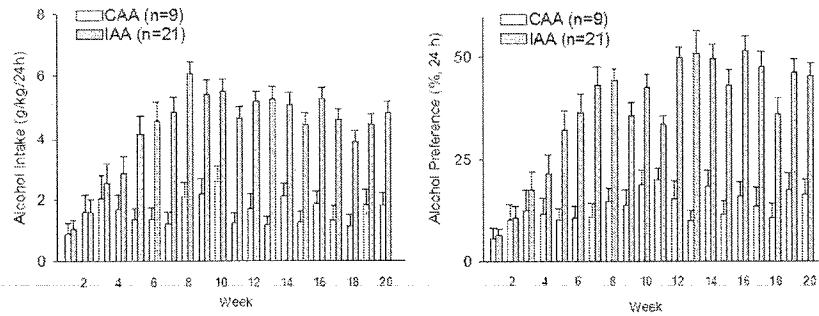

FIG. 4

the initial alcohol access period to 24 weeks, when we will re-initiate quinine adulterant testing and use a lower initial quinine concentration (0.003 g/l). We anticipate that this extended period of alcohol access will provide evidence of compulsive-like alcohol drinking (i.e., lack of suppression of drinking in response to adulteration of the alcohol with quinine, as demonstrated in previous studies) in some of the IAA rats but none in the continuous alcohol access rats, so the study can then be completed as originally planned.

This study is thus approximately 50% complete.

- **SPECIFIC AIM 2 (Objective 2): Evaluate PTSD/alcohol interactions,** *providing information from rat models that will likely reveal especially promising bases for:*

a) determining cause-effect between AUDs and vulnerability to developing PTSD **(TASK 4)**

STATUS: This work compares production of a PTSD-like behavioral and acoustic startle profile in rats with *vs* without a previous recent history of alcohol liquid diet-induced excessive prolonged alcohol intake. The study is underway; initial results are illustrated in **Fig. 5**. Rats which had chronically consumed liquid diet containing 7.5% ethanol (EtOH) ad libitum (n=18), or which were pair-fed isocaloric liquid diet with no ethanol (PF; n=18) received either traumatic stress (TS; 10 seconds inescapable footshock) or non-stress (NS) at 5 hour after removal of the liquid diet. After weekly 1-minute contextual reminders of the TS or NS in this rat PTSD model, each rat was tested for acoustic startle response (ASR) in the dark and again with bright illumination, as described in the proposal. Rats that had not consumed alcohol (i.e., pair-fed rats, PF) exhibited increased ASR in both the dark and light following TS + reminders (PF+TS), compared to PF rats that had not received TS (PF+NS) (p<0.01 for dark and for light), as predicted in this rat PTSD model. Furthermore, EtOH+NS rats exhibited increased ASR compared to
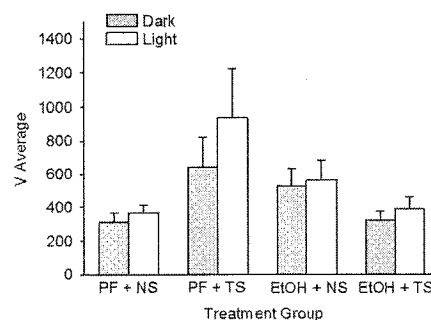

FIG. 5

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PF+NS rats, as predicted. However, EtOH+TS rats unexpectedly tended to exhibit decreased dark and light ASR relative to EtOH+NS, suggesting that TS during alcohol withdrawal decreased expression of the these indices of PTSD. We speculate that the results were probably confounded because the rats still had elevated circulating levels of alcohol at the time of the TS (presumably due to slow and sustained absorption of alcohol from the high fat/high sugar liquid diet that remained in the stomach even after removing access to the liquid diet. Accordingly, we are repeating this experiment with administration of TS vs NS at 12 hours after removal of liquid diet (when other liquid diet studies have demonstrated maximal expression of withdrawal symptoms), rather than the 5 hours used in the study our protocol was modeled after.

Although this experiment was completed, it may have been confounded and thus needs to be re-done with incorporation of the noted minor procedural change.

b) predicting who, among individuals with PTSD, is especially vulnerable to developing AUDs **(TASK 5)**.

STATUS: This work addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent acquisition of increased IAA alcohol intake. This work corresponds to **Task 5**. Nonetheless, it is notable that this work was initiated with the study addressing Task 6 (discussed below). These studies are being done in parallel because the methods and time schedules are compatible, with the major difference being that the study addressing Task 6 also incorporates pharmacologic treatment to decrease noradrenergic signaling at the time of traumatic stress. This is work is approximately 75% complete.

c) predicting who, among individuals with PTSD, is likely to respond to prazosin with decreased alcohol drinking.

STATUS: This work addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent effectiveness of prazosin treatment in suppressing IAA alcohol intake. **This work was not identified as a separate specific Task, but it is a component of several of the proposed experiments**. As described in the proposal, prazosin is being administered prior to voluntary alcohol drinking in rats that previously have been characterized for acoustic startle and anxiety-like behaviors in each of the experimental models used in these studies; we continue to evaluate whether prazosin treatment disproportionately decreases alcohol drinking in those rats with pre-existing or PTSD-induced high acoustic startle and high anxiety-like behavior. It is notable that some of this work has been initiated with the study addressing Task 6 (discussed below). As with the previous study, these experiments are being done, in part, in parallel because the methods and time schedules are compatible, with the major difference being that the study addressing Task 6 also incorporates pharmacologic treatment to decrease noradrenergic signaling at the time of traumatic stress. This work is

approximately 75% complete. Nonetheless, some initial high impact results have already been discussed in **TASK 1b**, illustrated in Fig. 1.

  ▪ **SPECIFIC AIM 3 (Objective 3): Determine whether the reduction of α1-AR mediated signaling at the time of traumatic stress will prevent the subsequent development of increased alcohol abuse and PTSD,** *informing whether prophylactic prazosin treatment is likely to decrease vulnerability to PTSD and alcohol use disorders* **(TASK 6)**.STATUS: This work includes pharmacologic reduction of noradrenergic signaling at the time of traumatic stress to determine whether this treatment blocks subsequent development of a rat PTSD-like behavioral and acoustic startle profile, as well as increased subsequent IAA alcohol intake. Recent results in some of our unrelated studies (funded by a separate NIH grant) have revealed that the combination of prazosin + the β-adrenergic antagonist, propranolol, suppresses alcohol drinking and some behavioral responses by alcohol-preferring (P) rats (which, as discussed in our original proposal, exhibit many characteristics similar to PTSD) more effectively than either drug alone, so we tested responses to prazosin treatment as planned, but also included a comparison group receiving prazosin + propranolol treatment at the time of traumatic stress. Results of trials with 141 male Wistar rats are described here. The rats received a single traumatic stress (TS, 10 sec inescapable footshock) or nonstress (NS, 10 sec exposure to shock environment, but without administration of shock) followed by weekly contextual reminders (R) of the TS or NS, as described in detail in the original proposal. Either prazosin (1.5 mg/kg), prazosin (1.5 mg/kg) + propranolol (5 mg/kg), or vehicle alone were administered by intraperitoneal (IP) injection 30-45 minutes before the TS or NS and again at 2 hours after the TS or NS.

After 4-5 weekly R followed by 3-4 additional weeks of behavioral testing, the rats were allowed to voluntarily drink alcohol in an IAA model (20% ethanol *vs* water 2-bottle choice access for 24 hours/day on 3 non-consecutive days/week, as discussed in detail in the original grant proposal) for a total of 12 IAA trials (i.e., 3 IAA trials/week for 4 weeks). Alcohol intake was determined in the first 1

FIG. 6

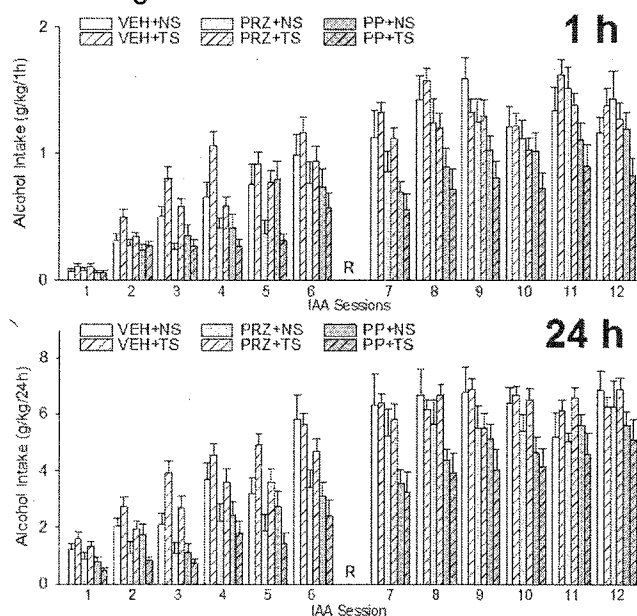hour as well as in all 24 hours of each IAA session. The results for the

first hour of drinking (thought to reflect motivation to drink alcohol for its acute pharmacologic effects, rather than drinking for caloric content and other factors affecting 24 h drinking) and for all 24 hours of each IAA session are shown in **Fig. 6**. Alcohol intake in the first hour as well as in all 24 hours of each IAA trial increased gradually but irregularly in the 12 successive IAA trials ($p<0.001$ by 2-way ANOVA with repeated measures on IAA trial), consistent with the gradual increase in alcohol drinking previously reported for the IAA model. A relatively consistent treatment response pattern in the first 6 IAA sessions (i.e., the first 2 weeks, when alcohol intake was progressively increasing) was qualitatively different from the consistently elevated alcohol intake and consistent response pattern in IAA 7-14 (i.e., weeks 3-4). Consequently, subsequent analyses were then conducted after averaging data for each rat over sessions 1-6 and 7-14. **Fig. 7** illustrates suppression of IAA alcohol intake and alcohol preference by prazosin (PRZ) or prazosin+propranolol (PRZ+PRO) treatment only at the time of the single TS/NS exposure 8-12 weeks earlier.
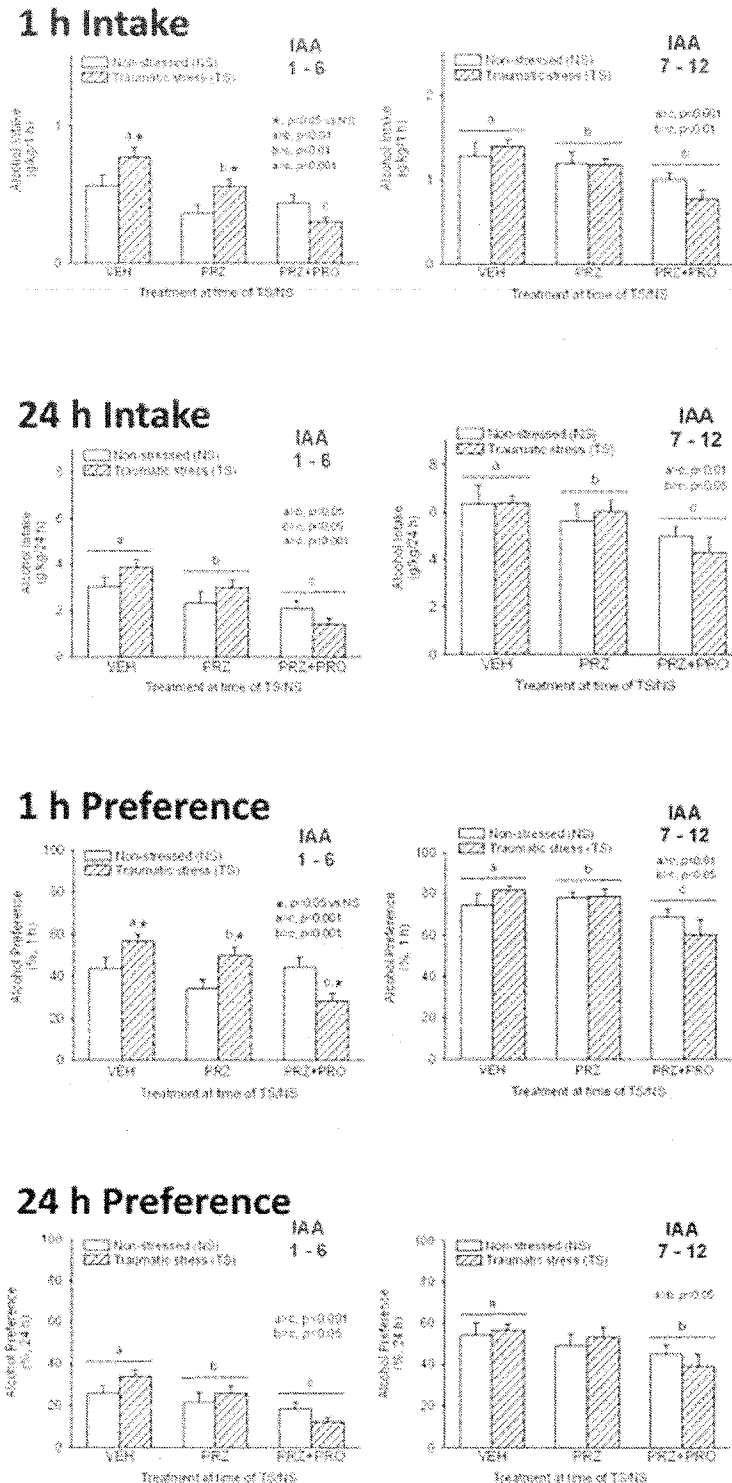
Fig 7, Row 1: **Alcohol intake during the first hour of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL).** In IAA trials 1-6 (LEFT PANEL), 1 h alcohol intake in rats that had received non-stress treatment (NS) 8 weeks prior to initiation of IAA was not significantly different among rats that had received VEH vs PRZ vs PRZ+PRO treatment at the time of the NS treatment. TS treatment 8 weeks prior to initiation of IAA increased 1 h alcohol intake ($p<0.05$ vs NS) in rats that had been treated with VEH or PRZ at the time of the TS exposure. The 1 h alcohol intake in rats that had received TS was suppressed by PRZ treatment at the time of TS ($p<0.05$ vs VEH), and further suppressed by PRZ+PRO treatment ($p<0.01$ vs PRZ alone). In IAA trials 7-12 (RIGHT PANEL), average alcohol drinking during the first 1 h of each IAA was suppressed by PRZ+PRO ($p<0.001$ vs VEH, $p=0.01$ vs PRZ) treatment at the time of the single TS/NS exposure, independent of TS/NS exposure. Each bar represents data from 15-35 rats.
A preliminary study demonstrated that PRO (5 mg/kg) at the time of TS did not suppress (vs VEH) either 1 h or 24 h alcohol intake during IAA initiated 8 weeks later (data not shown).

Fig. 7, Row 2: **Alcohol intake during the entire 24 hours of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL).** Average 24 h alcohol intake during IAA trials 1-6 (LEFT PANEL) was suppressed by PRZ treatment ($p<0.05$ vs VEH) - and further suppressed by PRZ+PRO treatment ($p<0.05$ vs PRZ, $p<0.001$ vs VEH) - at the time of the single TS/NS exposure 8 weeks earlier, independent of TS vs NS exposure. In IAA trials 7-12 (RIGHT PANEL), 24 h alcohol intake was suppressed by PRZ+PRO ($p<0.01$ vs VEH, $p<0.05$ vs PRZ) treatment at the time of the single TS/NS exposure, independent of TS vs NS exposure. Each bar represents data from 15-35 rats.

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Fig. 7, Row 3: Alcohol preference during the first hour of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL). In the <u>first 6 IAA trials (LEFT PANEL)</u>, 1 h alcohol preference in rats that had received non-stress treatment 8 weeks prior to initiation of IAA were not significantly different among rats that had received VEH *vs* PRZ *vs* PRZ+PRO treatment at the time of the NS treatment. TS treatment 8 weeks prior to initiation of IAA increased 1 h alcohol preference ($p<0.05$ *vs* NS) in rats that had been treated with VEH or PRZ at the time of the TS

1 h Intake

24 h Intake

1 h Preference

24 h Preference

FIG. 7

exposure; in contrast, TS at 8 weeks prior to IAA decreased alcohol preference ($p<0.05$ *vs* NS) in rats that had received PRZ+PRO at the time

of the TS exposure. The 1 h alcohol preference in rats that had received TS was suppressed by PRZ+PRO treatment at the time of TS ($p<0.001$ vs VEH or PRZ). In IAA trials 7-12 (RIGHT PANEL), average 1 h alcohol preference in rats was suppressed by PRZ+PRO ($p<0.01$ vs VEH or PRZ) treatment at the time of the single TS/NS exposure, independent of prior TS/NS exposure. Each bar represents data from 15-35 rats. A preliminary experiment demonstrated that PRO (5 mg/kg) at the time of TS did not suppress either 1 h or 24 h alcohol preference during IAA initiated 8 weeks later. A separate experiment demonstrated that PRZ+PRO treatment at the time of TS did not alter 1 h intake of 1% sucrose in the week following completion of IAA (data not shown).

Fig. 7, Row 4: Alcohol preference during the entire 24 hours of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL). Average 24 h alcohol intake during IAA trials 1-6 (LEFT PANEL) was suppressed by PRZ+PRO treatment ($p<0.001$ vs VEH, $p<0.05$ vs PRZ), independent of TS vs NS exposure. In IAA trials 7-12 (RIGHT PANEL), 24 h alcohol intake was suppressed by PRZ+PRO ($p<0.05$ vs VEH) treatment at the time of the single TS/NS exposure, independent of TS/NS exposure. Each bar represents data from 15-35 rats.

These results suggest that a single traumatic stress followed by weekly contextual reminders of the stress can increase voluntary alcohol drinking by male Wistar rats ≥ 8 weeks after the traumatic stress.

Treatment with the α1-adrenergic antagonist prazosin (PRZ) or – especially – combination treatment with both PRZ and the β-adrenergic antagonist propranolol (PRO) to reduce noradrenergic signaling at the time of the traumatic stress can decrease or prevent this later development of increased alcohol drinking, even though the drugs were administered only at the time of the traumatic stress (i.e., 30 minutes before and again 2 h after the stress) whereas the increased alcohol drinking was expressed ≥ 8 weeks later. These results suggest that PRZ or PRZ+PRO (or other treatments that decrease noradrenergic signaling) at the time of traumatic stress could potentially provide prophylaxis for alcohol use disorders that commonly accompany development of PTSD. In contrast, a preliminary study demonstrated that PRO alone did not suppress the TS-induced IAA alcohol drinking.

The doses and times of PRZ or PRZ+PRO administration before traumatic stress in this study were the same as the doses and times that we previously demonstrated to acutely decrease alcohol drinking in rats without producing sedating or motor effects.

Trauma memory testing before the IAA was initiated, at 8 weeks after the single traumatic shock (TS), confirmed that the TS + reminders (R) rats remembered the single TS and that the context of the TS remained aversive (expressed as increased avoidance behavior and increased defecation during R, each $p<0.01$ relative to NS, results not

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shown). These results suggest that PRZ treatment at the time of TS can decrease subsequent development of increased voluntary alcohol drinking 8-12 weeks after TS, that this response to PRZ is markedly enhanced by co-treatment with PRO, and that these responses are probably not mediated by decreasing the perceived aversiveness of the TS or by decreasing memory of the TS.

PRZ or [PRZ+PRO] administration at the time of TS potentially may provide effective preventive treatment for TS-induced development of alcohol abuse and – perhaps – PTSD or other correlates of PTSD. Further investigations are warranted to address effects of treatments at other time points relative to TS and R, resolution of responses by TS-sensitive vs -resilient subjects, and mechanisms in order to identify potentially clinically-effective interventions.

This work was presented at the 2016 Annual Meeting of the Research Society on Alcoholism and is now in preparation for publication. We will also analyze the extensive acoustic startle and behavioral test results as discussed in the original proposal, addressing potential correlations and predictive validity of characterizations before and after the rat PTSD model in determining development of a PTSD-like condition and/or increased alcohol intake. This overall Task is thus approximately 75% complete.

- **CHANGES, PROBLEMS, DELAYS AND PLANS TO RESOLVE THEM**
There are no significant changes in objectives and scope.

As discussed in previous reports, a change from the original proposal was the incorporation of osmotic minipumps for long-term drug administrations. However, as now discussed for Task 2, unanticipated problems with this method now require that this method be abandoned and the experiment for Task 2 be re-done using the originally proposed method of repetitive oral administration. We are now requesting to improve this original method by administering the alpha-1 adrenergic receptor, doxazosin, rather than prazosin. Doxazosin has the same receptor specificity as prazosin, we have previously demonstrated that it suppresses rat alcohol drinking consistent with prazosin, and our colleague and consultant, Dr. Murray Raskind, has demonstrated that, like prazosin, it decreases PTSD symptoms in clinical studies. Since doxazosin has a longer metabolic half-life than prazosin, it will maintain alpha-1 adrenergic receptor antagonism better than prazosin when administered on a repetitive daily basis.

The order of the studies was changed in the Year 1 progress report and in the SOW revised at that time; at present all studies are in progress, although it is now necessary to re-do some experiments to address problems, as discussed in this progress report.

As also previously noted in the Years 1 and 2 reports, it took longer than anticipated to recruit, hire (6 months after the award notice), process

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and train one new staff member (Shelby Johanson) in the first year, introducing delays at the start of the project. In Year 2, my long-time Laboratory Manager/Research Scientist, Carrie Kincaid, left my lab for a higher paying position as Research Manager for an extensive clinical research program at this VA medical center. After having her working with me for 10 years, this was of course highly disruptive. We then recruited, hired, processed and trained her replacement, Jennifer Burns, but she left the lab after 6 months when she received a fellowship to enter a neuroscience PhD program in Pittsburgh. Consequently, this year we again hired, processed and trained her replacement, Kristen Baumann, who has proven to be an effective addition to the lab. Nonetheless, these repetitive personnel changes and the time that it has taken to get the new personnel up to speed and operating efficiently has caused problems and delayed progress, so much so that we now have entered a 1-year no-cost extension period. We anticipate that this extension will allow effective completion of all studies, including sufficient time for appropriate thorough analysis, presentation and publication of results.

- **SUMMARY DISCUSSION**

The first year was used for personnel recruitment and training, implementation of all necessary methodologies, completion of the first study (which is key to interpreting all subsequent studies), and initiating subsequent studies - each of which is an individually long-term study (ranging from months to greater than a half-year for each of multiple temporally-overlapping cohorts of subjects within each study). In the second year another large key study progressed well and initial results were enthusiastically received when presented at the annual meeting of the International Society of PsychoNeuroEndocrinology, a meeting that was focused on effects of stress on the brain. The final cohorts of animals have now completed all trials in this study and analyses of interactions of PTSD-like responses with alcohol drinking are underway. Some results from this final complete complement of animals were presented this year at the 2016 Annual Meeting of the Society for Research on Alcoholism and are currently in preparation for publication. Further analyses of behavioral and physiological predictive indices of rat PTSD will now further extend the impact of this complete study, as planned. All remaining studies are underway and the overall project remains on track, although there have been delays due to repeated personnel changes and to the need to repeat some of the studies due to technical and methodological problems. Nonetheless, with the additional time of the 1-year no-cost extension we anticipate effective and thorough completion of these important and high impact studies.

4. KEY RESEARCH ACCOMPLISHMENTS

- The first key new accomplishment in Year 3 is the completion of the full complement of experimental trials completing our demonstration that pharmacologic reduction of noradrenergic signaling at the time

of a single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress and long after the brief pharmacotherapy at the time of the trauma. This finding is important because it reveals a potential prophylactic approach in preventing effects of traumatic stress on subsequent alcohol drinking. Perhaps even more important, it also reveals additional potential clinical interventions that now can be readily tested with the model that we have developed, such as prevention of PTSD responses by suppressing noradrenergic signaling at other time points (e.g., in the time period immediately following a traumatically stressful experience to block development of PTSD or sequelae, or at the time of reminders to facilitate extinction therapy).

- The second key new accomplishment in Year 3 is our demonstration that the effect of acute pharmacologic reduction of noradrenergic signaling on increased voluntary alcohol drinking is dependent on the individual rat's history of previous stresses. This finding is important because it informs clinical trials in the path to implementing prazosin or other noradrenergic treatments to decrease alcohol abuse, since there has been large individual variability in responses to prazosin and doxazosin. We plan to address this important issue in a symposium for which the PI is co-organizer, proposed for the 2017 Annual Meeting of the Research Society on Alcoholism.

- Additional new findings in Year 3 are key to the successful completion of the remaining studies, even though they have introduced additional delays. First, the use of osmotic minipumps for chronic constant administration was determined not to be effective because the solutions of prazosin tended to precipitate out over time, preventing administration of the drug. Although this requires that we now repeat a study, we have an alternative approach (repetitive oral administration of doxazosin - which has a longer half-life that allows daily administration – in a piece of sweetened gelatin containing the doxazosin that the rats readily consume that we have used in previous studies). Second, administration of traumatic stress at 5 hours after removal of liquid diet containing alcohol, a model of previous alcohol abuse effects on response to traumatic stress, was found to be inappropriate. This too requires that another completed experiment needs to be repeated, but with a longer (i.e., 9-12 hour) interval between removal of liquid diet and subsequent administration of traumatic stress, which we have previously determined to reliably be associated with significant alcohol withdrawal symptoms.

5. CONCLUSION:

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The key results previously reported for Year 1 were consistent with the hypothesis that is central to all other studies in this research project, i.e. that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to development of increased alcohol drinking. These results provided the conceptual basis for a potential approach to prospectively identifying individuals – including individuals with PTSD - at increased risk for future alcohol use disorders, thus allowing development and implementation of potential preventive interventions.

The key result from a large experiment started in Year 2 and completed in Year 3 now provides evidence for a promising potential preventive intervention. We have previously demonstrated that a single episode of traumatic stress in our rat PTSD model can produce sustained marked increases in hyper-responsiveness reflected in acoustic startle response, which is noradrenergic activation dependent; the key result for Year 2, i.e., that reduction of noradrenergic signaling at the time of single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress, demonstrates a potential pharmacologic intervention for preventing at least the subsequently increased alcohol drinking following a traumatic stress. As further behavioral data from the complete study soon become available, we will also evaluate what other aspects of PTSD-like rat behavior also respond to this pharmacologic intervention. The new key finding then also suggests subsequent related questions, such as "would treatment only immediately after a trauma also be effective?", or "would treatment only at the time of each contextual reminder also be effective?", and "what mechanisms are involved?" Addressing these subsequent questions would inform potentially effective pharmacotherapy in cases where traumatic stress had already recently occurred, or perhaps as an adjunct to subsequent PTSD psychotherapy. Together, these two initial key results from also provide the conceptual bases for potential prospective identification of individuals – including individuals with PTSD – at increased risk for future alcohol use disorders, and then potentially applying preventive and – possibly - therapeutic pharmacologic intervention.

An additional key result revealed that the response to reduction of noradrenergic signaling depended on previous history of stress exposure. Rats that had been exposed to traumatic stress and subsequent contextual reminders in the rat PTSD model subsequently exhibited increased voluntary IAA alcohol drinking, and administration of prazosin or prazosin+propranolol acutely suppressed this drinking. However, rats that had not experienced traumatic stress did not exhibit such elevated IAA drinking and this voluntary alcohol drinking was not significantly suppressed by prazosin or prazosin+propranolol. These results inform - and provide a pre-clinical model for further investigating – the

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interpretation of variable responses to suppression of noradrenergic signaling that have been reported in clinical investigations.

The remaining studies, which are all in progress, will likewise further develop a model that will be useful for current and future investigations of neurobiological mechanisms mediating initiation and development of excessive drinking, mechanisms mediating co-morbidity of alcohol use disorders and PTSD, and additional potential treatments of both.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS
a. Manuscripts
Lay press: Nothing to report

Peer-Reviewed Scientific Journals:
Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-naïve male rats predicts subsequent voluntary alcohol intake and alcohol preference. Alcohol Alcohol. 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065; PMID: 25305255)

No new papers to report in the third year; two are currently in preparation.

Invited Articles: Nothing to report

Published abstracts:
Rasmussen DD, Johanson SS, Kincaid CL. Reduction of $\alpha 1$-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. Psychoneuroendocrinology 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421) Included in appendix.

Rasmussen DD, Johanson SS, Burns JL, Kincaid CL. Reduction of $\alpha 1$- and β-adrenergic signaling at the time of traumatic stress reduces subsequent development of increased alcohol drinking in rats. Alcohol.Clin.Exp.Res. 40 (S1):198A, 2016.

b. Presentations:
The PI and a PTSD clinical investigator colleague and consultant on this project, Dr. Murray Raskind, together presented a joint seminar at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC) and the VAPSHCS division of Research and Development in affiliation with the Seattle Institute of Biomedical and Clinical Research (SIBCR), entitled "Translation goes both ways; prazosin treatment from humans to rats and back". This presentation was not published.

The PI presented the work described in the appended published abstract to the 44th Annual Meeting of the International Society for

PsychoNeuroEndocrinology – Stress and the Brain, on September 10, 2015. Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. Psychoneuroendocrinology 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421)

The PI presented work described in an appended published abstract to the 2016 Annual Meeting of the Research Society on Alcoholism: Rasmussen DD, Johanson SS, Burns JL, Kincaid CL. Reduction of α1- and β-adrenergic signaling at the time of traumatic stress reduces subsequent development of increased alcohol drinking in rats. Alcohol.Clin.Exp.Res. 40 (S1):198A, 2016. PMID not assigned.

7. **INVENTIONS, PATENTS AND LICENSES**: Nothing to report

8. **REPORTABLE OUTCOMES:** Our initial key finding in Year 1, that acoustic startle in alcohol-naïve rats is highly predictive of subsequent voluntary IAA alcohol drinking and preference, extends and complements the results of one of our previous studies demonstrating that pre-stress acoustic startle predicts development of rat PTSD-like further increased acoustic startle and plasma corticosterone response following a traumatic stress. The key finding from preliminary findings in Year 2, maintained in completion of the entire experiment in Year 3 (in which the number of trials were nearly doubled) demonstrated that reduction of noradrenergic signaling at the time of a single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress, extends and complements our Year 1 key finding by demonstrating a potential new pharmacotherapeutic approach for preventing at least the subsequently increased alcohol drinking following a traumatic stress. Together these results suggest that increased acoustic startle and associated increased anxiety - both of which are increased by noradrenergic activation - reflect underlying mechanisms that increase vulnerability to both PTSD and alcohol abuse. Together with our and others' previous results demonstrating that prazosin can decrease both voluntary alcohol intake and PTSD symptoms, these results strongly suggest that prazosin can be effective for both conditions and that an α1-adrenergic receptor-mediated mechanism is at least one component of the common underlying mechanism, and thus an especially appropriate target for both prophylactic and therapeutic interventions. However, another key finding in Year 3 further refines this interpretation by demonstrating that the voluntary alcohol drinking response to decreasing noradrenergic signaling appears to be dependent upon prior history of traumatic stress experience. Although this complicates interpretations of the overall results, it is consistent with the evidence that all clinical subjects do not respond equally to prazosin or doxazosin treatment. Our studies provide an effective preclinical model for further resolving this variability in responses, prospectively identifying who is most likely to respond, and

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developing new treatments, or effective treatment combinations (such as prazosin or doxazosin administered together with naltrexone, which we demonstrated in studies - supported by a separate NIH grant – to suppress alcohol drinking in female alcohol-preferring (P) rats even when prazosin alone did not). The remaining studies further investigate these interactions, facilitating most effective translation of prazosin treatment to clinical utility. All studies are now in progress and, although there have been setbacks due to unexpected personnel turnovers and to methodologic issues that have made it necessary to now repeat some experiments, these studies are nonetheless progressing well to successful completion. Our further development of the rat PTSD model, employing a single traumatic stress together with weekly brief contextual reminders of the stress will – together with the further characterization of PTSD-like responses in these studies – also provide a well-characterized experimental model for other labs investigating PTSD and alcohol abuse, alone or together. In addition, a) the findings, results and techniques of these studies are directly applicable to other investigations of the effects of stress or the evaluation of mechanisms contributing to voluntary alcohol and other drug abuse, b) the results of this investigation will facilitate translating prazosin (and doxazosin) treatment to clinical implementation in the treatment of PTSD and alcohol abuse, alone or together, and c) our results will ultimately improve overall understanding and effective treatment of alcoholism and PTSD, two conditions with profound negative social and economic impact.

9. **OTHER ACHIEVEMENTS:** A great deal of basic and clinical PTSD and related traumatic brain injury (TBI) research is based at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC), providing ample ongoing collaborative opportunities. The PI works closely with a PTSD clinical investigator colleague, Dr. Murray Raskind, as well as alcohol clinical investigators (Drs. Andrew Saxon and Tracy Simpson) and a TBI clinical investigator, Dr. Elaine Peskind, within the VAPSHCS and MIRECC to facilitate translation of basic science findings in the current investigation to clinical testing and future clinical implementation as discussed in Section 8.

10. REFERENCES

Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-naïve male rats predicts subsequent voluntary alcohol intake and alcohol preference. Alcohol Alcohol. 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065; PMID: 25305255)

Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development

of increased alcohol drinking. Psychoneuroendocrinology 61: 53, 2015
(doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421)

Rasmussen DD, Johanson SS, Burns JL, Kincaid CL. Reduction of α1-
and β-adrenergic signaling at the time of traumatic stress reduces
subsequent development of increased alcohol drinking in rats.
Alcohol.Clin.Exp.Res. 40 (S1):198A, 2016. PMID not assigned.

11. **APPENDICES:** Two published abstracts and one published paper, listed
above in section 6, are appended.

cal pathways involved in sexual desire and function. This paper will present preliminary data suggesting that known neuroendocrine correlates of PTSD are associated with SD. In combat veterans with PTSD ($n = 76$), loss of sexual interest was associated with lower levels of the adrenal androgen DHEA ($\beta = .316$, $t72 = -2.364$, $p = .021$) and plasma cortisol ($\beta = -.378$, $t72 = -3.344$, $p = .001$), and with an attenuated response to the dexamethasone suppression test ($\beta = -.314$, $t67 = -2.565$, $p = .013$). Heightened catecholamines as reflected by the NE/cortisol ratio were associated with problems during sexual relations ($r = .382$, $p = .028$, $df = 31$) and also predicted PTSD intrusive symptoms ($\beta = .216$, $t89 = 2.04$, $p = .044$). In a separate study of treatment seeking veterans with PTSD, urinary NE was associated with difficulty achieving orgasm ($\beta = .340$, $t39 = 2.34$, $p = .024$). In a subsample analysis, testosterone levels did not distinguish SD, suggesting that sexual problems were not the result of organic disorder. Implications and avenues for future research will be discussed.

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PO86

HPA axis dysregulation in patients with hypersexual disorder

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Recent focus has been raised on hypersexual disorder that was suggested as a diagnosis for the DSM-5. However, little is known about the neurobiology behind this disorder. A dysregulation of the hypothalamic pituitary adrenal (HPA) axis has been shown in psychiatric disorders but it is not investigated in hypersexual disorder. The aim of this study was to investigate the function of the HPA axis in hypersexual disorder.

The study includes 67 male patients with hypersexual disorder and 39 healthy male volunteers. Basal morning plasma levels of cortisol and ACTH were assessed and low dose (0.5 mg) dexamethasone suppression test was performed with cortisol and ACTH measured post dexamethasone administration. Non-suppression status was defined with DST-cortisol levels ≥138 nmol/l. The Sexual Compulsive scale (SCS), Hypersexual disorder current assessment scale (HD:CAS), Montgomery-Åsberg Depression Scale-self rating (MADRS-S) and Childhood trauma questionnaire (CTQ), were used for assessing hypersexual behavior, depression severity and early life adversity.

Patients with hypersexual disorder were significantly more often DST non-suppressors, had significantly higher DST-ACTH levels and their DST-Cortisol levels showed a trend to be higher compared to healthy volunteers. The patients reported significantly more childhood trauma and depression symptoms compared to healthy volunteers. CTQ scores showed a significant negative correlation with DST-ACTH whereas SCS and HD:CAS scores showed a negative correlation with baseline cortisol in hypersexual patients. The diagnosis of hypersexual disorder was significantly associated with higher plasma DST-ACTH even when adjusted for childhood trauma.

The results suggest a possible HPA axis dysregulation in patients with hypersexual disorder.

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Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking

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Activation of α1-adrenergic receptor (AR)-mediated mechanisms contributes to post-traumatic stress disorder (PTSD) and to increased acoustic startle response. Pre-stress acoustic startle response (ASR) predicts subsequent increased startle response in a rat PTSD model in which a single traumatic stress (TS, 10 second footshock) is followed by weekly contextual reminders (R) of the TS. ASR also predicts development of increased alcohol drinking in a rat intermittent alcohol access (IAA) model (20% alcohol access for 24 h/day on 3 non-consecutive days/week). We thus hypothesized that reduction of α1-AR signaling at the time of TS would prevent subsequent development of the increased alcohol drinking that commonly accompanies development of PTSD. Male Wistar rats received either TS or no-shock (NS). After 4 weekly R, IAA was provided for 4 weeks. During the final week, average alcohol intake in the first hour of each IAA was increased ($p < 0.05$) in [TS + R] vs [NS + R] rats. The α1-AR antagonist, prazosin (PRZ; 1.5 mg/kg), administered IP at the time of the single TS decreased the [TS + R]-induced IAA alcohol drinking ($p < 0.05$, relative to treatment with vehicle), as did PRZ + the β-AR antagonist, propranolol (PRO, 5 mg/kg). These results suggest that PRZ or PRZ + PRO treatment at the time of TS can prevent subsequent development of increased alcohol drinking. We will further address effects of PRZ or PRZ + PRO administered at other time points relative to TS and R, and potential roles of corticosterone. Supported by VA Puget Sound Health Care System, Seattle, Washington and by US Army Medical Research CDMRP W81XWH-13-1-0126.

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PO88

Is salivary estriol detectable in very early pregnancy?

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Background: Estriol is produced in large quantities by the placenta during pregnancy and is important for early pregnancy maintenance. In maternal blood, estriol can be detected from the 8th week of pregnancy and increases sharply after the 10th week under the influence of the HPA axis hormone ACTH. Although estriol can be reliably analyzed in saliva from the 13th week, informa-

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REDUCTION OF α1- AND β-ADRENERGIC SIGNALING AT THE TIME OF TRAUMATIC STRESS REDUCES SUBSEQUENT DEVELOPMENT OF INCREASED ALCOHOL DRINKING IN RATS

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We previously demonstrated that pre-stress acoustic startle response (ASR) predicts subsequent further increased ASR in a rat PTSD model in which a single traumatic stress (TS) is followed by repeated weekly contextual reminders (R) of the TS. We also demonstrated that ASR predicts subsequent development of increased voluntary alcohol drinking in rats. Since noradrenergic activation contributes to increased ASR, anxiety, alcohol drinking, and PTSD, we hypothesized that reduction of noradrenergic signaling at the time of TS would decrease the subsequent development of increased alcohol drinking that commonly accompanies development of PTSD and increased anxiety. Male Wistar rats received either TS (10 sec footshock) or no-shock (NS). After 4 weekly R, intermittent alcohol access (IAA; choice between water and 20% alcohol for 24 h/day on 3 nonconsecutive days/week) was provided for 4 weeks. Combined IP administration of the α1-adrenergic antagonist, prazosin (PRZ, 1.5 mg/kg), and the β-adrenergic antagonist, propranolol (PRO, 5 mg/kg), at the time of the single 10 sec TS decreased average alcohol intake by 46% in the first hour of each IAA, 8–12 weeks after the TS ($p < 0.001$, relative to treatment with vehicle). Administration of PRZ (1.5 mg/kg) alone at the time of the single TS also decreased average alcohol intake ($p < 0.001$, relative to vehicle), but only by 32%, which was less than the suppression by [PRZ+PRO] ($p < 0.01$). Alcohol preference was likewise suppressed more by [PRZ+PRO] than by PRZ alone ($p < 0.01$). Trauma memory testing before the IAA was initiated at 8 weeks after the single TS demonstrated that the TS+R rats remembered the single TS and that the context of the TS remained aversive (expressed as increased avoidance behavior and increased defecation during R, each $p < 0.01$ relative to NS). These results suggest that PRZ treatment at the time of a TS can decrease subsequent development of increased voluntary alcohol drinking 8–12 weeks after TS, that this response to PRZ is enhanced by co-treatment with PRO, and that these responses are not mediated by decreasing the perceived aversiveness of the TS or by decreasing memory of the TS. PRZ or [PRZ+PRO] administration at the time of TS potentially may provide effective preventive treatment for TS-induced development of alcohol abuse and – perhaps – PTSD or other correlates of PTSD. Further investigations are addressing effects of treatments at other time points relative to TS and R, resolution of responses by TS-sensitive versus -resilient subjects, and mechanisms. Supported by VA Puget Sound Health Care System, Seattle, WA and by US Army Medical Research CDMRP W81XWH-13-1-0126.

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IMPACT OF CHRONIC ETHANOL SELF-ADMINISTRATION ON KAPPA OPIOID RECEPTOR REGULATION OF DOPAMINE SIGNALING IN NONHUMAN PRIMATES

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Although alcoholism is one of the most prevalent disorders in the United States, with over 18 million individuals meeting the criteria for an alcohol use disorder, the exact neurobiological bases of this condition remain obscure. Recently, it has been demonstrated that kappa-opioid receptor (KOR) signaling in the striatum plays a critical role in the increased reinforcing efficacy of ethanol following ethanol vapor exposure in rodent models. Here we examined the effects of chronic voluntary ethanol self-administration in macaques on dopamine neurotransmission and the ability of KORs to regulate dopamine release in the nucleus accumbens core. Three cohorts of nonhuman primates were given free access to 4% ethanol (w/v) for 22 h/day. These cohorts were composed of male cynomolgus, female rhesus or male rhesus macaques, and were given access to ethanol for 6, 12, or 18 months, respectively. Ex vivo fast-scan cyclic voltammetry was then conducted the nucleus accumbens core to determine dopamine signaling kinetics as well as the ability of U50,488 (KOR agonist) to inhibit dopamine release. We found that chronic ethanol drinking increased dopamine uptake rates, which could have implications for reductions in basal dopamine tone in vivo during ethanol withdrawal. Further, across sex, strain and exposure length, ethanol use augmented the ability of KORs to inhibit dopamine release, demonstrating that ethanol-induced increases in KOR sensitivity are widespread and independent of other factors. Finally, KOR sensitivity was positively correlated with lifetime ethanol intake, suggesting that changes in KOR regulation of dopamine release may be a determinant of aberrant drinking behaviors. Nonhuman primate models of ethanol abuse represent a highly translational avenue for identifying molecular targets for pharmacotherapeutic compounds, and here we show, for the first time, that voluntary ethanol self-administration has a unique effect on KOR sensitivity and regulation of dopamine release directly at the dopamine terminal that was positively correlated with drinking behavior. Together, these data provide novel insight into ethanol-induced dysregulation of opioid signaling and suggest that dopaminergic dysfunction may be mediating increases in voluntary drinking. Importantly, KOR antagonists may be efficacious in reducing drinking behaviors in alcoholics.

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TAAR1 ACTIVATION PREFERENTIALLY DECREASES PHASIC DOPAMINE RELEASE AND DECLINES ETHANOL SEEKING BEHAVIOR IN RATS

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Recent studies suggest that trace amine-associated receptor 1 (TAAR1) may represent a novel therapeutic target for the treatment of several neuropsychiatric disorders, including addiction. However, the mechanisms involved in the psychotropic actions of TAAR1 agonists are still unclear. The present study explored the effects of a partial TAAR1 agonist, RO5263397, on presynaptic dopamine (DA) transmission, using optogenetics combined with real-time DA detection. We also assessed the effect of this compound on operant ethanol drinking behavior. We applied a new viral technology to restrict the expression of ChR2 to DA cells in the VTA of Long Evans rats, driving ChR2-EYFP expression via a tyrosine hydroxylase promoter. The viral construct was microinjected into the VTA of rats ($n = 5$) and DA release was measured by fast-scan cyclic voltammetry in anesthetized rats. Importantly, the level of ChR2 expression was sufficient to allow us to optogenetically mimic tonic and phasic patterns of accumbal DA release. The experiments indicated a significant effect of RO5263397 (10 mg/kg, i.p.) on the amplitude of the optogenetically-induced DA signal ($p < 0.0001$). Notably, the compound differentially affected phasic and tonic patterns of DA release. Thus, the drug decreased phasic DA release more powerfully than tonic DA efflux ($p < 0.05$). Since there are marked regional variations in DA dynamics between striatal and accumbal terminals, we explored local effects of RO5263397 on DA efflux in the nucleus accumbens core, shell and dorsal striatum. The study was performed on brain slice preparations, where a single pulse (4 ms) of electrical stimulation was applied to evoke DA transients. High concentrations of the compound significantly decreased DA release in the nucleus accumbens core ($p < 0.05$ for 50 μM and $p < 0.0001$ for 100 μM) and dorsal striatum ($p < 0.0001$ for 100 μM). There was a significant difference in the effects of the compound at 50 and 100 μM between regions ($p < 0.05$). Specifically, electrically-evoked DA release in the shell region was less responsive to RO5263397. These in vitro data suggest that RO5263397 at high concentrations can affect presynaptic DA receptors at the level of terminals (preferentially, in the nucleus accumbens core and dorsal striatum). Finally, the effects of the compound on ethanol drinking behaviors were studied using the operant self-administration procedure. The rats were trained to press a lever 30 times daily for 20 min access to 10% ethanol. After subjects displayed stable appetitive and consummatory behaviors (≈ 6 weeks), the effect of RO5263397 (5 or 10 mg/kg, i.p.) or saline were evaluated. Pretreatment with the TAAR1 agonist significantly suppressed lever press behaviors at both doses tested with a main effect of treatment ($p < 0.001$). Importantly, when access to ethanol was provided, no significant changes in consummatory measures (e.g. number of licks, ethanol intake) were observed. In conclusion, these results suggest that the TAAR1 agonist tested selectively suppresses ethanol seeking behaviors, presumably through the inhibition of phasic DA release in the nucleus accumbens. This proposes the fascinating possibility that TAAR1 agonists may be considered as promising candidates for the treatment of alcohol addiction.

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DHM EFFECTS ON ETHANOL-INDUCED DOPAMINE RELEASE IN THE RAT NUCLEUS ACCUMBENS

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Direct and indirect involvement of the inhibitory GABA-A and glycine receptors has previously been demonstrated to be of importance for the ability of ethanol (EtOH) to increase dopamine levels in the nucleus accumbens (nAc). In a series of studies, we suggested that EtOH primarily acts in the nAc, where it triggers a neuronal circuitry involving nAc glycine receptors as well as ventral tegmental nicotinic acetylcholine receptors, in order to elevate extracellular accumbal dopamine. We have also investigated a number of substances known to decrease EtOH intake in rodents and/or humans, alone or in combination with EtOH with regards to influence on nAc dopamine. In the present study we aimed to explore the influence of dihydromyricetin (DHM) alone and in combination with EtOH on extracellular levels of dopamine in the nAc, as measured by in vivo microdialysis in freely moving Wistar rats. Since EtOH previously was demonstrated to increase endogenous levels of the amino acid taurine, a ligand to both GABA-A and glycine receptors, and DHM previously was suggested to mediate its EtOH reducing effects via the GABA-A receptor we also measured taurine, glycine, serine and glutamate in the dialysate samples. After systemic administration of DHM we found a modest (approximately 20%) increase of nAc dopamine whereas no alteration of any of the measured amino acids was detected. In rats pretreated with DHM a systemic injection of EtOH (2.5 g/kg i.p. 20 min after DHM) failed to produce any further dopamine elevation. In the present study DHM, a substance with potential interest for treating human alcohol use disorders, was found to display a similar pattern with regards to nAc dopamine as previously found for several other investigated compounds (acamprosate, varenicline and the glycine uptake inhibitor Org-25935). Thus, since yet another compound with affinity for inhibitory receptors in the nAc was demonstrated to produce a modest dopamine increase and prevent EtOH from further increasing extracellular dopamine levels we suggest that this drug profile is beneficial in the purpose of controlling excessive EtOH intake in humans.

Acoustic Startle in Alcohol-Naïve Male Rats Predicts Subsequent Voluntary Alcohol Intake and Alcohol Preference

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Abstract — Aims: Acoustic startle response in rats is used to model sensorimotor reactivity. The aim of the study was to determine whether acoustic startle response in alcohol-naïve rats predicts subsequent increased voluntary alcohol drinking or alcohol preference. **Methods:** Startle responses to 90, 95 and 100 decibel (dB) white noise stimuli presented in counterbalanced semi-randomized order were tested in alcohol-naïve young adult male Wistar rats before voluntary alcohol intake was established with an intermittent alcohol access (IAA) model. **Results:** Startle amplitude in response to 95 or 100 dB stimuli was positively correlated with subsequent alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split) pre-IAA startle amplitude in response to 95 or 100 dB stimuli developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA, relative to rats with low pre-IAA startle amplitude. **Conclusion:** Startle response to moderate acoustic stimuli can be a predictive index of vulnerability to developing increased alcohol drinking.

INTRODUCTION

Begleiter and Porjesz (1999) proposed that sensorimotor hyper-reactivity is a key feature of the simplest model of the neuronal milieu underlying a predisposition to alcoholism. This hypothesis is consistent with evidence that sensorimotor hyper-reactivity expressed as enhanced acoustic startle response is characteristic of abstinent alcoholics (Krystal *et al.*, 1997) and is associated with family history of alcoholism (Pfefferbaum *et al.*, 1991; Grillon *et al.*, 1997). Rats selectively bred for alcohol preference and high voluntary alcohol drinking (McKinzie *et al.*, 2000; Chester *et al.*, 2004; Acewicz *et al.*, 2012), and post-dependent rats experiencing either acute alcohol withdrawal or prolonged imposed alcohol abstinence (Rassnick *et al.*, 1992; Rasmussen *et al.*, 2005) also exhibit increased acoustic startle response.

Enhanced startle is associated with increased brain noradrenergic activation (Stevens *et al.*, 1994), and brain noradrenergic activation contributes to increased voluntary alcohol drinking (Walker *et al.*, 2008; Rasmussen *et al.*, 2009; Simpson *et al.*, 2009; Froehlich *et al.*, 2013; O'Neil *et al.*, 2013). Enhanced startle is also correlated with the increased anxiety (Morgan *et al.*, 1993; Davis *et al.*, 1997) that is common to many alcoholics (Cloninger, 1987; Kushner *et al.*, 2000) and that is a major risk factor for alcohol abuse (Koob and Le Moal, 1997). Furthermore, anxiety-related behavior in rats has been demonstrated to predict alcohol drinking under several schedules of alcohol access (Hayton *et al.*, 2012). We thus hypothesized that characterization of startle response may facilitate prospective identification of vulnerability to developing increased voluntary alcohol drinking and also may provide a basis for determining mechanisms mediating development of some alcohol use disorders. Accordingly, we investigated whether prospectively determined acoustic startle response in alcohol-naïve rats was correlated with subsequent increased voluntary alcohol drinking or increased alcohol preference in an intermittent alcohol access (IAA) model. IAA, in which rats have access to 2-bottle choice (water vs 20% alcohol) home cage alcohol drinking for three 24-h sessions/week, separated by at least 24 h (e.g. Monday, Wednesday, Friday),

has been reported to induce outbred Wistar rats to escalate alcohol intake over repetitive access sessions to achieve alcohol intake at individually variable high levels accompanied by high alcohol preference and blood alcohol concentrations (BACs) comparable to those achieved by selectively bred alcohol-preferring (P) rats, and has been suggested to effectively model some human alcohol use disorders (Wise, 1973; Simms *et al.*, 2008).

MATERIALS AND METHODS

Animals

Twenty-three alcohol-naïve young adult male Wistar rats (Simonsen Labs, Gilroy, CA, USA) weighing 285 ± 3 g were housed 2/cage in plastic shoebox cages with controlled temperature (21 ± 1°C) and a 12 h/12 h light/dark cycle (lights off at 0900 h). Standard rodent chow (Laboratory Rodent Diet #7001, Harlan Teklad, Madison, WI, USA) and water were available *ad libitum* throughout the study. All experimental procedures were approved by the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Acoustic startle testing system

Acoustic startle was tested with an SR-LAB Acoustic Startle System (SDI, San Diego, CA, USA) using a slight modification of methods we previously reported (Rasmussen *et al.*, 2008). Each SR-LAB test chamber includes a ventilated sound-attenuated cabinet containing a clear plastic cylindrical rat enclosure mounted on a piezoelectric accelerometer that detects muscle twitch in response to a brief pulse of white (mixed frequency) noise produced by a tweeter inside the cabinet. The force exerted on the accelerometer is digitized and expressed in millivolt (mV) units. Each startle response signal generated by the accelerometer was recorded as 65 consecutive 1 ms recordings, starting at the onset of each 40-ms startle tone. Results were analyzed as maximum peak

amplitude. Startle stimulus and background white noise levels were calibrated with a Radio Shack Digital Sound Level Meter (33–2055; RadioShack Corp., Fort Worth, TX, USA) placed in the center of the cylindrical rat enclosure. Each of 8 SR-LAB test chambers was calibrated before each use to provide 450 mV response to a consistent test stimulus provided by an SDI Standardization Unit (SDI, San Diego, CA, USA). Each SR-LAB test chamber and rat enclosure was cleaned with 0.5% Liquinox (Alconox, Inc., New York, NY, USA) before each use.

Pre-test acclimation

After acclimation to the animal colony room and the reversed light/dark cycle for 3 weeks, rats were transferred to a dark testing room adjoining the colony room for 90 min at 1.5–3 h after lights-off on each of 5 days prior to testing, with ambient 60 decibel (dB) white noise produced by a White Noise Generator (SDI, San Diego, CA, USA). On the first 4 days, each rat was acclimated to a dark SR-LAB test chamber for 5 min with 60 dB white background noise (produced by the tweeter inside the chamber) before being returned to the colony room, housed with the same cage mate. On the fifth day, each of the rats was likewise transferred to the dark testing room with ambient 60 dB white noise, placed in the dark SR-LAB test chamber for 5 min with 60 dB white background noise, and then exposed to 10 presentations of 40 ms 95 dB white noise pulses presented at 30 s intervals before being returned to the colony room. The goal of this initial exposure to acoustic pulses was to minimize effects of novelty stress in the subsequent acoustic startle testing with similar white noise pulses. Immediately following completion of the acclimation process, each rat was returned to the same colony room but individually housed in a plastic shoebox cage. All procedures during the acclimation and subsequent acoustic startle testing and IAA were conducted under dim red illumination.

Acoustic startle testing

Startle testing was conducted 7–10 days after completion of the pre-test acclimation. On the test day, rats were again transferred to the dark testing room with 60 dB background white noise. After 90 min, each rat was placed in a dark SR-LAB testing chamber with 60 dB background white noise for 5 min before quantitation of startle responses to 10 presentations each of 40 ms 90, 95 or 100 dB white noise pulses at 30 s intervals, with one pulse of each intensity (i.e. 90, 95 or 100 dB) in

Table 1. Order of acoustic stimuli presentations

Sequential sets of 3 acoustic stimuli	Counterbalanced order of stimuli within sets (dB)		
1	90	95	100
2	95	100	90
3	100	90	95
4	90	95	100
5	95	100	90
6	100	90	95
7	90	95	100
8	95	100	90
9	100	90	95
10	90	95	100

There were 30 s intervals between stimuli within each set as well as between each sequential set.

counterbalanced order within each of 10 sequential sets of three pulses (Table 1), with 60 dB background white noise between pulses. Thus, there were a total of 30 startle tests at 30 s intervals, with 10 tests/each of responses to 90, 95 or 100 dB pulses distributed in counterbalanced order over a 15-min period.

Alcohol drinking

Three weeks after startle testing, 2-bottle choice access to 20% (v/v) alcohol vs water was provided for 24 h/day, 3 days/week (M, W, F)—i.e. an IAA model (Wise, 1973; Simms et al., 2008). The alcohol solution was prepared by diluting 95% alcohol (ethanol; Decon Labs, King of Prussia, PA, USA) with deionized water to make a 20% (v/v) solution. Alcohol (20%) and water were presented in ball-bearing sipper tubes, with positions of the tubes alternated in sequential alcohol access periods to control for potential side preferences. On days when alcohol was not provided, the rats had access to water only. On days when alcohol and water intakes were characterized for analysis, daily fluid intakes were determined by weighing each tube to the nearest 0.1 g. Alcohol and water tubes were also placed on two empty cages to determine loss due to spillage/leakage and evaporation; average losses in these two cages on each day were subtracted from intakes for that day. Net daily alcohol intake was converted to g alcohol/kg body weight. After 36 alcohol access days (i.e. 12 weeks, when stable alcohol intake was achieved) alcohol intake and alcohol preference (ml of alcohol intake/[ml of alcohol intake + ml of water intake]) were determined over the next 3 alcohol access days to characterize the relationships of each rat's alcohol intake and alcohol preference relative to its pre-IAA acoustic startle responses. One rat did not establish significant daily alcohol drinking (alcohol intake was <1 g/kg/day on all days evaluated) and was excluded from further analyses.

Data analyses

Startle amplitude in response to presentations of 90, 95 or 100 dB acoustic pulse intensities in counterbalanced order within each of 10 sequential sets of 3 pulse presentations were initially evaluated by two-way (set X pulse intensity) repeated measures analysis of variance (ANOVA) with repeated measures on sets (1–10) and pulse intensities (90, 95 or 100 dB). There was a significant effect of intensity, $F(2, 42) = 63.7$, $P < 0.001$, but no significant effect of set and no significant intensity × set interaction. Since startle amplitude in response to each of the acoustic stimulus intensities was independent of presentation time (set) within the 15 min test period, the average of all 10 responses to each stimulus intensity was used in subsequent analyses of the relationships between pre-IAA acoustic startle response vs IAA alcohol intake or alcohol preference. Similarly, IAA alcohol intake or alcohol preference on the 3 alcohol access days in IAA week 13 was analyzed by one-way ANOVA with repeated measures on day; there were no significant effects of day on either alcohol intake or alcohol preference, so 3-day average alcohol intake or 3-day average alcohol preference was likewise used in subsequent analyses of relationships between IAA week 13 alcohol intake or alcohol preference vs pre-IAA acoustic startle amplitude.

Pre-IAA startle amplitude in response to presentations of either 90, 95 or 100 dB stimuli was each compared with subsequent alcohol intake or alcohol preference by Pearson Product Moment Correlation Analysis. The pre-IAA startle response to

90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11$/group) IAA week 13 alcohol intake was further compared by two-way (high vs low alcohol intake X stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The pre-IAA startle amplitude in response to 90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11$/group) IAA week 13 alcohol preference was likewise compared by two-way (high vs low alcohol preference X stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The IAA week 13 alcohol intake or alcohol preference of rats grouped on the basis of high vs low (median split, $n = 11$/group) pre-IAA startle amplitude in response to either 90, 95 or 100 dB stimuli was each analyzed by Student t-test (median splits of startle responses to each of the three stimulus intensities did not in each case identify the same animals to be included in the high vs low startle response groups, so two-way ANOVA testing could not be performed); Bonferroni corrections were not applied to individual t-tests.

All analyses were conducted using Sigmaplot Version 11 software (Systat Software, Inc., Chicago, IL, USA) with significance accepted at $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

IAA alcohol intake and alcohol preference

Initial (i.e. IAA week 1) average (M, W, F) alcohol intake was 0.95 ± 0.16 g/kg/24 h and alcohol preference was 0.07 ± 0.01. By IAA week 13, alcohol intake had increased to 3.80 ± 0.32 g/kg/24 h ($P < 0.001$) and alcohol preference had increased to 0.40 ± 0.03 ($P < 0.001$).

IAA alcohol intake relative to pre-IAA startle response

IAA week 1 alcohol intake was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.18$), 95 ($P = 0.11$) or 100 ($P = 0.15$) dB stimuli. IAA week 13 alcohol intake relative to pre-IAA startle amplitude elicited in response to presentations of either 90, 95 or 100 dB stimuli is presented in the upper, middle or lower row, respectively, of Fig. 1.

Pre-IAA startle amplitude in response to 90 dB stimuli was modest and inconsistent; startle amplitude was not significantly correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, upper row, left). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol intake revealed no alcohol intake-dependent significant difference in pre-IAA startle in response to 90 dB stimuli (Fig. 1, upper row, center; in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) x stimulus intensity (90, 95,100 dB)] interaction, $F[2, 40] = 5.1$, $P \le 0.01$, but pre-IAA startle amplitude in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol intake (Fig. 1, upper row, right).

Pre-IAA startle amplitude in response to 95 dB stimuli was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, middle row, left; $P < 0.01$, $r =$

0.62). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol intake in IAA week 13 (Fig. 1, middle row, center; $P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle amplitude in response to 95 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 95 dB stimuli (Fig. 1, middle row, right; $P \le 0.001$).

Pre-IAA startle amplitude in response to 100 dB stimuli also was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, lower row, left; $P < 0.01$, $r = 0.55$). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, center; $P < 0.001$). Consistent with this results, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, right; $P < 0.01$).

IAA alcohol preference relative to pre-IAA startle response

IAA week 1 alcohol preference was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.19$), 95 ($P = 0.14$) or 100 ($P = 0.20$) dB stimuli.

IAA week 13 alcohol intake and alcohol preference were highly positively correlated, $r = 0.92$, $P < 0.001$. Further analyses of IAA week 13 alcohol preference relationships to pre-IAA startle responses were conducted identically to those in the preceding analysis of alcohol intake relationships to pre-IAA startle responses. Consistent with the high positive correlation between alcohol intake and alcohol preference, the results of analyses of alcohol preference vs pre-IAA startle responses, as detailed below, were essentially identical to the results of the preceding analyses of alcohol intake vs pre-IAA startle responses. Pre-IAA startle amplitude in response to 90 dB stimuli was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.05$, $r = 0.47$). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol preference revealed no alcohol preference-dependent significant difference in pre-IAA startle response to 90 dB stimuli (in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) x stimulus intensity (90, 95, 100 dB)] interaction, $F[2, 40] = 6.41$, $P < 0.01$, but pre-IAA startle in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol preference.

Pre-IAA startle amplitude in response to 95 dB pulses was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.57$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.05$). Consistent with this result, rats with high (median split) pre-IAA startle response to 95 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA low startle response to 95 dB stimuli ($P < 0.01$).
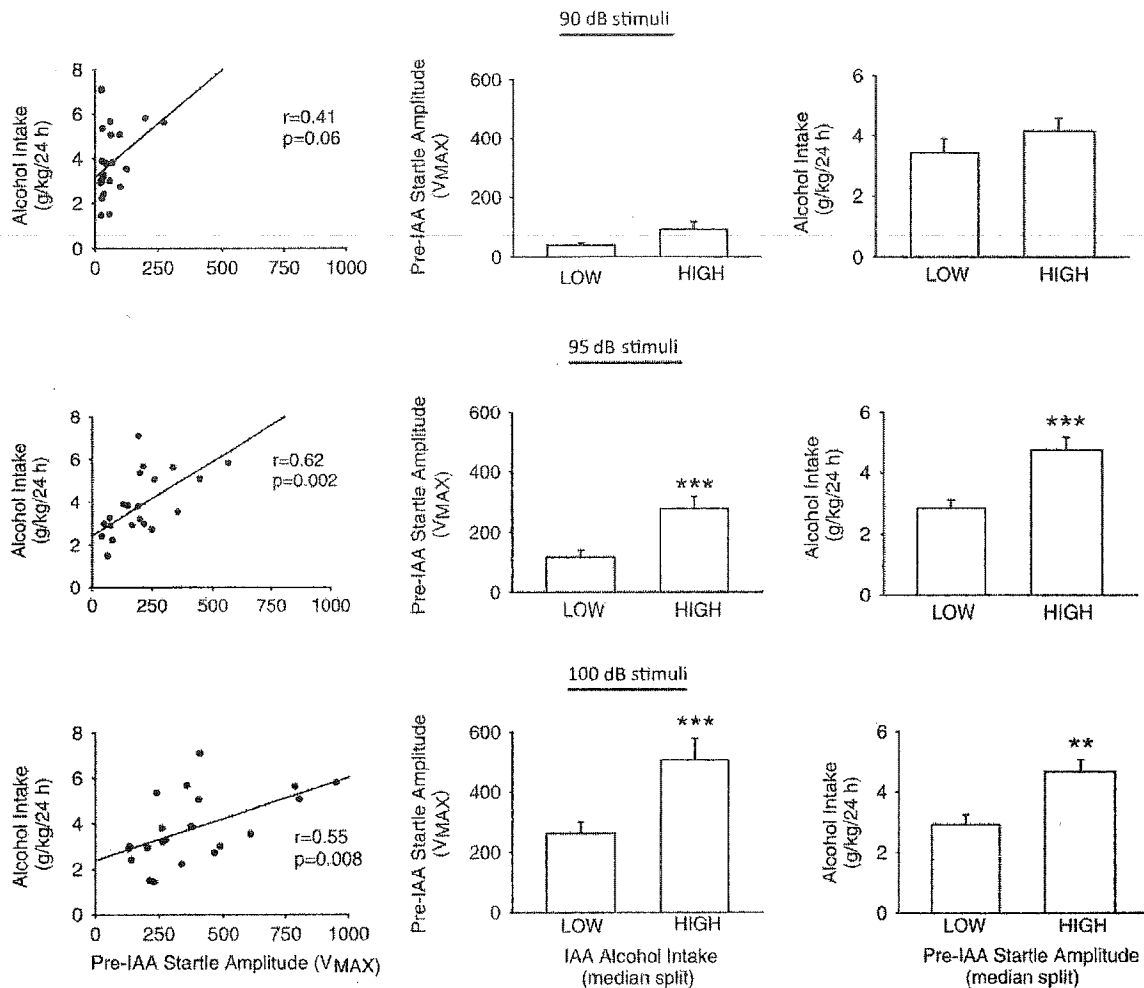
Fig. 1. Pre-IAA acoustic startle response vs alcohol intake following 3 months of IAA. Rows: The upper, middle and lower rows present analyses of pre-IAA responses to 90, 95 or 100 dB acoustic startle stimuli, respectively. Columns: The left panel in each row presents the correlation between pre-IAA acoustic startle amplitude vs IAA week 13 alcohol intake for all 22 rats. The center panel in each row presents the pre-IAA acoustic startle amplitude of rats grouped on the basis of low vs high (median split, $n = 11$ rats/group) alcohol intake in IAA week 13. The right panel in each row presents the IAA week 13 alcohol intake of rats grouped on the basis of low vs high (median split, $n = 11$ rats/group) pre-IAA acoustic startle amplitude. $**P \le 0.01$ vs Low, $***P \le 0.001$ vs Low.

Pre-IAA startle response to 100 dB stimuli also was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.53$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli ($P < 0.01$).

IAA alcohol intake relative to pre-IAA startle response to the first presentation of each stimulus intensity

Some previous investigations of alcohol drinking in rats have compared alcohol intake or preference relative to startle

amplitude in response to only the first presentation of an acoustic stimulus. In the current study, IAA week 13 alcohol intake or alcohol preference was not significantly correlated with pre-IAA startle in response to the first presentation of either 90 or 100 dB stimuli. However, pre-IAA startle amplitude in response to the first 95 dB stimulus was correlated with IAA week 13 alcohol intake ($r = 0.66$; $P \le 0.001$) as well as alcohol preference ($r = 0.56$; $P < 0.01$).

DISCUSSION

In alcohol-naïve young adult male Wistar rats, acoustic startle amplitude in response to 40 ms pulses of white noise at intensities of 95 or 100 dB was positively correlated with subsequent voluntary alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split)

alcohol intake or alcohol preference following the 3 months of IAA had previously exhibited greater pre-IAA startle response to 95 as well as 100 dB stimuli, relative to rats with low alcohol intake or low alcohol preference. Conversely, rats with high (median split) pre-IAA startle response to 95 or 100 dB stimuli subsequently developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA.

Stimulus intensities in this investigation were based on the results of preliminary trials with young male Wistar rats in which 90 dB stimuli produced inconsistent small startle responses, 95 or 100 dB stimuli reliably produced relatively consistent sub-maximal startle, and a higher intensity stimulus (120 dB) produced maximal responses. The moderate 90, 95 and 100 dB stimuli were selected in order to avoid ceiling effects that could compromise ability to differentiate responses between animals, as suggested by a report that human startle amplitudes elicited by 90 dB, but not 114 dB, stimuli were positively correlated with number of previous alcohol detoxifications (Krystal et al., 1997). It previously has been reported that male Wistar rats exhibited an inverted U-shaped curvilinear relationship between the startle response to an initial 120 dB acoustic stimulus vs later alcohol intake, and that startle habituation appeared to have predictive value regarding alcohol intake (Sandbak et al., 2000). In the current study, habituation to repeated stimulus exposures was not apparent, and there were significant positive linear correlations between pre-IAA acoustic startle responses to 95 or 100 dB stimuli vs alcohol intake and alcohol preference following IAA. The apparent disparities between the Sandbak et al. (2000) study and the current study may be due to the differing stimulus intensities as well as to the incorporation of an initial session with exposure to repetitive moderate (95 dB) stimuli in advance of the testing trial in the current study in order to minimize novelty of the stimulus (consistent with clinical studies, in which the subjects are aware that they will hear acoustic stimuli during the testing trial). In addition, stimuli of three different intensities were presented in semi-random counterbalanced order throughout the 15 min trial in the current study, rather than consistent repetition of a single stimulus. It is also notable that the current study used an IAA model of alcohol drinking in which a relatively high concentration of alcohol (20%, v/v) was available on 3 intermittent days each week, considered to be a model for excessive alcohol drinking (Wise, 1973; Simms et al., 2008).

Alcohol-naïve rats from lines selectively bred to prefer alcohol exhibit increased acoustic startle relative to selectively bred alcohol non-preferring rats (McKinzie et al., 2000; Chester et al., 2004; Acewicz et al., 2012). Sons of alcoholics likewise exhibited increased acoustic startle compared with sons of non-alcoholic parents (Grillon et al., 1997). The current results suggest that mechanisms contributing to acoustic startle response have a functional role in the vulnerability to increased voluntary alcohol drinking, and that acoustic startle characterization can provide an index of sensorimotor hyper-reactivity and associated mechanisms that contribute to this increased alcohol drinking. Although these mechanisms remain to be resolved, it has been demonstrated that brain noradrenergic activation increases acoustic startle response (Stevens et al., 1994) and also produces sensorimotor hyper-reactivity and anxiety (Redmoond and Huang, 1979; Sullivan et al., 1999) which are major risk factors for development of

alcohol use disorders (Cloninger, 1987; Koob and Le Moal, 1997; Begleiter and Porjesz, 1999; Kushner et al., 2000). Conversely, suppression of noradrenergic signaling not only decreases acoustic startle responses (Gresack and Risbrough, 2011; Olson et al., 2011) but also decreases alcohol drinking in rats and humans (Walker et al., 2008; Rasmussen et al., 2009; Simpson et al., 2009; Froehlich et al., 2013; O'Neil et al., 2013) and blocks the expression of increased alcohol drinking in rats selectively bred for alcohol intake (Froehlich et al., 2013). The consistent association of changes in acoustic startle, anxiety and increased alcohol drinking with changes in noradrenergic signaling suggests that noradrenergic activation may have a key role in mediating the correlation between acoustic startle amplitude and subsequent development of increased voluntary alcohol drinking.

The current results demonstrate that acoustic startle amplitude in response to moderately supra-threshold startle stimulus intensities administered to alcohol-naïve male Wistar rats is an effective predictive index for subsequent increased voluntary alcohol intake and alcohol preference in the IAA model. Acoustic startle response may be an especially useful index of the vulnerability to developing increased alcohol drinking because it is not dependent upon, and potentially confounded by, interactions with other behaviors. Importantly, acoustic startle is also well-characterized for use in humans (Krystal et al., 1997; Grillon and Baas, 2003; Grillon et al., 1998, 2005), providing translational utility.

These results may provide a useful model for investigating neurobiological mechanisms mediating initiation and development of excessive alcohol drinking, as well as provide the conceptual basis for a potential approach to prospectively identifying individuals at increased risk for future alcohol use disorders, thus allowing potential preventive intervention.

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